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Toxicology Testing of the Unique Radiolytic Product 2-Dodecylcyclobutanone

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Abstract

Treatment of foods containing fatty acids, including meat and poultry, with ionizing radiation can lead to the formation of a class of chemicals called 2-alkylcyclobutanones (2-ACBs) that are unique to irradiated foods. The major 2-ACB formed in irradiated meat is 2-dodecylcyclobutanone (2-DCB), which is formed by the radiolysis of palmitic acid. The U.S. Food and Drug Administration (FDA) recommends that indirect food additives consumed in quantities greater than 1.5 μg per day be tested for safety. On average, approximately 6.0 μg of 2-DCB is present in an irradiated, and then cooked, 125 g ground beef patty, which exceeds the 1.5 μg/day limit. Because of the availability of irradiated ground beef as part of the National School Lunch Program in the U.S. "consumer groups" opposed to food irradiation have requested that 2-DCB be tested in appropriate genotoxicity assays, even though irradiated meats have been extensively tested for safety in animal studies, and have been approved by the FDA for consumption by humans. In order to address the question of 2-DCB genotoxicity the purified compound was tested in 6 genotoxicity tests including bacterial reverse mutation assays, a 5-flouro-uracil mutagenesis assay, the yeast DEL assay, the Pro-Tox™ Assay, and for the formation of 6-thioguanine resistant mutants in human TK6 lymphoblasts. No 2-DCB induced mutagenesis was observed in any of the test systems, both with and without exogenous metabolic activation.

Introduction

The use of ionizing radiation to improve the microbiological safety of meat and poultry products has been controversial due to the misplaced association of food irradiation with atomic weapons, nuclear waste, and visions of accidents at nuclear power plants. Groups opposed to food irradiation are typically opposed to any type of nuclear technology, and cite the increased risk of cancer associated with exposure to ionizing radiation and nuclear contamination even though irradiated foods do not contain nuclear waste and are not radioactive. Consumers are simply uncomfortable with the word irradiation, despite many years of research that have failed to detect an increased risk of cancer or birth defects with long-term consumption of irradiated meat and poultry in feeding studies using multiple species of animals [1].

An issue that further increases the negative perception of irradiated foods is chemicals present in irradiated foods known as unique radiolytic products. Exposure of foods containing fatty acids, such as meat and poultry, to ionizing radiation leads to the formation of compounds called 2-alkylcyclobutanones (2-ACBs), which are not detectable in non-irradiated food products. The most abundant of the 2-ACBs in irradiated meat is 2-dodecylcyclobutanone (2-DCB), which is formed by cleavage of the acyl-oxygen bond of palmitic acid by ionizing radiation that leads to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid but with an alkyl group in the second ring position ($C_{16}H_{30}O$; FW 238.41) (Figure 1) [2]. A person consuming 125 g of cooked irradiated ground beef would be expected to consume approximately 6.0 μg of 2-DCB, or 0.00006 mg/kg for a 100-kg adult, or 0.00024 mg/kg for a 25-kg child [calculated from reference 3].

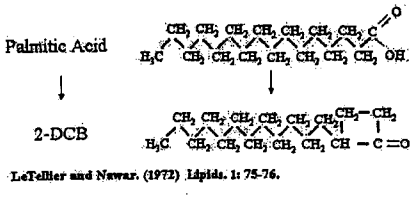

Fig. 1. Structure of Palmitic Acid and 2-Dodecylcyclobutanone.

While irradiated meat and poultry has been tested extensively for toxicological safety, unique radiolytic products such as 2-DCB have not been evaluated for safety in pure form. Methodology for the synthesis of 2-

DCB, and other 2-ACBs, that could generate sufficient quantities of the compound to complete an appropriate assessment of their genotoxicity has only been recently described, and is cost prohibitive due to their high cost (> $12, 000 per gram) [4].

Despite the high cost and difficulty of testing 2-DCB for genotoxicity Delincee and Pool-Zobel initiated such work in 1998 and found that 2-DCB concentrations of 0.125 to 1.25 mg/ml could induce DNA strand breaks in rat and human tumor cells *in vitro* [5]. However, these results were later found to be an artifact of the protocol that was used [6]. The Comet Assay sometimes yields false positive results due to chromosome fragmentation when cytotoxicity (cell death) is induced [5, 6]. When 2-DCB was retested at non-cytotoxic concentrations, no increase in DNA strand breakage was observed in human colon cell lines, but in the same study oxidative damage to DNA was detected. In a more recent study, Knoll et al. [7] obtained a 2-fold increase in DNA strand breaks and chromosome rearrangements using in primary human cells *in vitro* using the Comet Assay and the 23 Color Fluorescent *In Situ* Hybridization (FISH) Assay.

Recent work at the USDA Agricultural Research Service's Eastern Regional Research Center located in Wyndmoor, PA, has focused on a systematic assessment of 2-DCB's genotoxic potential. Genotoxicity testing of 2-DCB has included U.S. FDA recommended tests in addition to mechanistic studies in order to elucidate a possible reason for the inconsistency of test results, which includes either negative responses or a weakly genotoxic response, or an overall equivocal response.

Materials and Methods

Test Organisms: *Salmonella* Mutagenicity Test and *Escherichia coli* TRP Assay tester strains were obtained from Moltox, Inc. (Boone, NC, USA). *E. coli* strains used for the Pro-Tox™ assay and generation of 5-fluorouracil resistant mutants were obtained from Xenometrix Inc. (Boulder, CO, USA). *Saccharomyces cerevisiae* strain RS112 used in the yeast DEL Assay was obtained from Dr. Robert Schiestl (UCLA, Los Angeles, CA, USA). Human TK6 lymphoblasts were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA).

Test Protocols: *Salmonella* Mutagenicity Test and the *E.coli* TRP test were conducted using the Miniscreen™ format as previously described by Sommers [4] and Sommers and Schiestl [8]. Forward mutation frequency testing for 2-DCB induced generation of 5-fluorourcacil resistant mutants was performed according to Skopek and Thilly [9] and Sommers and Mackay [10]. Induction of DNA damage inducible genes using the Pro-Tox™ assay was performed using the protocol of Orser et al. [11] and Sommers and Mackay [10]. The yeast DEL Assay was performed as described by Sommers and Schiestl [8]. Generation of 6-thioguanine resistant mutants in human TK6 lymphoblasts was performed as described [12,13,14].

Chemicals and Reagents: The chemicals 2-dodecylcyclobutanone (2-DCB), 5-fluorouracil (5-FU), Methyl methanesulfonate (MMS), 2-nitrofluorene (2-NF) and 6-thioguanine (6-TG) were all obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The compound 2-aminoanthracene (2-AA) was obtained from Moltox, Inc. Positive control concentrations are listed in their respective publications. The solvent for 2-DCB was dimethylsulfoxide (DMSO), which was obtained from Sigma-Aldrich Chemical Company. Reagents used for exogenous metabolic activation including S9 fraction from Aroclor 1254 induced male Sprague-Dawley rats and associated buffers were obtained from Moltox, Inc.

Results and Discussion

The ability of 2-DCB to induce the mutations in the *Salmonella* Mutagenicity Test and *E. coli* TRP assay are shown in Table 1 and Table 2. Tester strains included TA100 and TA1535 which are used to detect the formation of point (missense) mutations, while tester strains TA98 and TA1537 are used to detect the formation of frame-shift mutations. *E. coli* tester strains included both the nucleotide excision repair proficient and deficient derivatives of the WP2 parent strain. 2-DCB did not induce mutations in the reverse mutation assays at concentrations up to 1.0-mg/ microtiter plate well (2 mg/ml or 2-g/kg). Positive controls included MMS, 2-NF and 2-AA, and the number of revertant colonies generated were consistent with previous studies [4, 8].

Another type of mutagenesis assay is the detection of forward mutations, as oppose to reverse mutations. In the 5-Flourouracil (5-FU)-resistance test mutants in *E. coli* or *Salmonella* are formed when a null mutation is fixed within the DNA sequence of the 0.551 kb uracil-phosphoribosyltransferase gene, which would normally convert the nucleoside analog 5-FU to a toxic metabolite within the bacterium. The DNA target available for mutagenesis in these forward mutation frequency assays is much larger than that in bacterial reverse mutation tests, an entire gene as opposed to a point mutation. Sommers and Mackay [10] exposed *E. coli* SF1 to 2-DCB in liquid medium, as opposed to the plate incorporation test. No increase in mutation frequency (formation of 5-FU-resistant colonies), was observed at 2-DCB concentrations up to 1 mg/ml (1 g/kg), with or without exogenous metabolic activation, as determined by Student's *t* test (n=3, α=0.05) (Table 3) [10].

Table 1. Induction of mutations in the *E. coli* Tryptophan Reverse Mutation Assay by 2-DCB.

Tester Strain	S9 Fraction	*Revertant Colonies per Well*[a] 0.00 mg	0.05 mg	0.10 mg	0.50 mg	1.00 mg	*Pos.Control*
WP2 [pKM101]	0%	4.11±0.99	5.55±1.64	7.56±1.75	4.78±1.33	5.22±1.57	146±14.7
	5%	2.11±0.11	1.89±0.29	1.89±0.48	1.11±0.40	1.67±0.19	32.4±0.73
WP2 *uvrA* [pKM101]	0%	8.11±2.73	6.33±3.23	7.00±2.52	9.00±2.65	9.44±3.23	141±9.45
	5%	9.22±1.37	8.44±2.50	9.44±2.50	8.67±0.69	9.22±0.69	116±2.67

[a]The number of *Trp*$^+$ revertant colonies per well represents the mean of three independent experiments (n=3) followed by the standard error of the mean for those values [See Ref. 4].

Table 2. Induction of mutations in the *Salmonella* Mutagenicity Test by 2-DCB.

Tester Strain	S9 Fraction	Revertant Colonies per Well[a] 0.00 mg	0.05 mg	0.10 mg	0.50 mg	1.00 mg	*Pos. Control*
TA98	0%	4.00±0.50	3.83±0.33	3.5±0.00	3.67±0.17	3.33±0.88	111±4.16
	5%	3.33±0.44	2.33±0.67	1.83±0.33	2.00±0.29	2.83±0.17	92.2±3.25
TA100	0%	16.2±1.64	16.2±1.30	19.2±0.67	17.0±0.76	16.8±1.83	159±8.26
	5%	13.2±3.09	9.67±1.59	16.2±4.32	13.7±1.17	14.2±3.17	205±4.49
TA1535	0%	3.50±0.29	1.50±0.29	2.17±0.17	2.83±0.17	3.50±0.76	126±4.07
	5%	2.16±0.67	1.17±0.44	1.50±0.29	1.67±0.73	1.33±0.44	91.2±7.91
TA1537	0%	2.00±0.29	1.83±0.44	2.00±0.76	2.17±0.17	1.33±0.60	54.4±5.84
	5%	1.17±0.33	0.50±0.29	1.67±0.73	1.00±0.29	1.50±0.29	42.8±1.59

[a]The number of *HIS*$^+$ revertant colonies per well represents the mean of three independent cultures (n=3) followed by the standard error of the mean for those values. [see Ref. 8].

Table 3. Induction of 5-fluorouracil-resistant mutants in *E. coli* SF1 exposed to 2-DCB.

2-DCB Conc.	Frequency ($\times 10^{-6}$) of 5-FU resistant mutants 0 mg/ml	0.13 mg/ml	0.25 mg/ml	0.50mg/ml	1.00 mg/ml	*Pos.Control*
No S9 fraction	0.74±0.17	1.10±0.33	0.96±0.87	0.83±0.16	0.79±0.17	12.6±2.88
2% S9 fraction	1.09±0.14	0.95±0.14	0.88±0.16	0.97±0.14	0.90±0.22	7.43±1.99

[a]Results were tabulated from 3 independent experiments. Positive control compounds were 130 μg/ml MMS without S9 fraction, and 10 μg/ml 2-AA with S9 fraction [see Ref. 10].

As reviewed in Sommers and Mackay [10] gene expression profiling has been used extensively for determination of chemical genotoxicity and is capable of detecting many genotoxins that are not detectable using reverse and forward bacterial mutation assays. The UmuDC proteins of *E. coli* allow DNA polymerases to bypass DNA adducts during DNA replication in an error prone DNA repair pathway, RecA protein is both a ssDNA binding protein and a regulator of SOS inducible DNA repair pathways in *E.coli,* Nfo (DNA endonuclease IV) is required for the repair of oxidative DNA damage, while DinD (OrfA/PyrE) protein is involved in DNA replication and resolution of recombination intermediates, and transcription of RNA from each of these genes is increased following exposure of *E. coli* to xenobiotics that induce DNA damage [10]. 2-DCB was not able to induce gene expression, as measured by increased B-galactosidase levels, at concentrations of 9, 18, 36, 63, 125, 250, 500 and 1000 μg/ml in *E. coli* SF1 strains containing the promoter/β-galactosidase reporter constructs (Table 4), with or without exogenous metabolic activation (n=3, a=0.05) as determined by Students *t* Test. However, a non-significant 4 fold increase in the expression from the *Nfo* gene promoter should be noted, which is in agreement with results presented by Burnouf et al. [6] in which some oxidative DNA damage was observed in human cells (Table 4).

Table 4. Induction of DNA damage inducible genes, as measured by promoter β-galactosidase fusions,in response to 2-dodecylcyclobutanone, with and without exogenous metabolic activation.

Reporter	S9	2-dodecylclobutanone μg/ml 0	125	250	500	1000	Pos. Control
dinD-lacZ	-	4.37±1.43	3.48±0.84	3.50±1.28	3.36±2.17	1.21±0.67	28.8±10.1
	+	5.11±0.30	2.08±0.79	1.74±0.65	1.69±0.62	4.80±2.03	54.4±17.5
recA-lacZ	-	17.4±6.12	13.9±5.54	12.3±6.08	13.5±5.61	13.8±8.68	57.3±19.3
	+	7.55±1.9	5.27±1.21	4.50±1.67	5.23±1.24	4.51±1.15	65.1±5.67
nfo-lacZ	-	3.95±1.77	3.58±0.85	2.83±0.44	1.84±0.40	21.0±9.01	87.7±20.5
	+	21.8±2.40	13.6±3.00	13.5±3.03	13.2±2.27	14.8±1.33	104±10.9
umuDC-lacZ	-	9.89±2.91	10.6±3.07	9.86±3.77	10.4±4.13	10.6±5.25	57.1±11.2
	+	3.87±1.65	2.94±1.64	3.10±1.42	1.73±0.82	2.10±1.57	48.0±5.42

Results were tabulated from three independent experiments. There was no statistically significant increase in 2-DCB induced -galactosidase determined by Students *t* test (n = 3, α = 0.05). 2-DCB concentrations of 9, 18 and36 and 63 μg/ml 2-DCB also failed to increase β-galactosidase activity (data not shown). [see Ref. 10].

The yeast (*Saccharomyces cerevisiae)* DEL assay measures a chemicals ability to induce genomic rearrangements by restoration of a nonfunctional duplication of the *his3* gene to functionality (*HIS3*$^{+}$) by intrachromosomal recombination and detects many carcinogens that are positive in the *Salmonella* Mutagenicity Test as well as those which are non-mutagenic carcinogens [8]. Both classes of carcinogens induce DEL recombination between the two copies of an internal duplication of the HPRT gene in human cells and between two copies of the *pun* (pink-eye-unstable) gene *in vivo* in mice [8]. Thus the assay represents a simple test system for predicting clastogenicity *in vitro.* No significant increase in DEL recombination was observed at 2-DCB concentrations up to 5 mg/ml (5 g/kg).

Table 5. Induction of intrachromosomal (DEL) recombination in *Saccharomyces. cerevisae* RS112 by 2-DCB.

2-DCB Conc.	0 mg/ml	0.63 mg/ml	1.25 mg/ml	2.5 mg/ml	5.0 mg/ml	*Pos. Control*
Rec. Freq.	0.72×10^{-4}	0.97×10^{-4}	0.62×10^{-4}	1.01×10^{-4}	1.04×10^{-4}	16.7×10^{-4}
Standard Error	0.15×10^{-4}	0.15×10^{-4}	0.18×10^{-4}	0.60×10^{-4}	0.67×10^{-4}	1.24×10^{-4}
% Viability	100%	85.3%	86.5%	76.9%	28.5%	54.8%

*Results were tabulated from three independent experiments. There was no statistically significant increase in 2-DCB induced intrachromasomal recombination as determined by Students t test ($n = 3$, $\alpha = 0.05$) [see Ref. 8].

One of the FDA "recommended" mutagenicity tests is the Mouse Lymphoma Assay, in which a mouse cell line or human TK6 lymphoblasts may be used. The assay is similar to the forward mutation frequency assay used earlier in that mutants resistant to a nucleoside analog, in this case 6-TG, are selected for. Human TK6 cells were used to avoid the criticism that mouse cells were used. Human cells were exposed to 2-DCB for 4 hrs, with and without exogenous metabolic activation. The microtiter plate method [12,13,14] was used to determine mutation frequency. Each experiment was independently conducted three times. Statistically significant differences were determined using Student's *t* test. The 2-ACB did not induce formation of 6-TG resistant mutants in the mouse lymphoma assay using human TK-6 lymphoblasts (Table 6) [14]. Cell viability was reduced to approximately 45% in cultures with and without exogenous metabolic activation, as determined by trypan blue exclusion, following the preliminary 4 hr exposure to 2-DCB. Cells treated with concentrations of 2-DCB greater than 0.62 μg/ml were not recoverable following centrifugation, most likely due to severe membrane damage following exposure to the extremely hydrophobic test compound.

Table 6. Generation of 6-thioguanine resistant mutants in human TK6 lymphoblasts exposed to 2-DCB.

	S9	0 μg/ml	18 μg/ml	36 μg/ml	62 μg/ml	*Pos. Control*
Mutation Freq. (10^{-6})	-	2.78±0.63	3.26±0.67	3.04±0.11	3.20±0.82	26.6±5.81
Mutation Freq. (10^{-6})	+	5.04±0.93	6.13±1.23	5.64±1.66	5.55±1.69	55.3±13.0

[a]Positive controls were significantly different from untreated controls as determined Students *t* Test ($n=3$, $\alpha=0.05$). [see Ref. 14]

Genotoxicity testing of 2-DCB, and most abundant 2-ACB in irradiated meats, has yielded either negative results, or a very weakly positive result (2 fold increase in DNA strand breaks or chromosome aberrations), in the mutagenicity and clastogenicity tests utilized to date. Testing in bacterial mutagenicity assays recommended by the U.S. FDA have yielded negative results. Mechanistic studies using the yeast DEL Assay and gene expression profiling in bacteria also yielded negative results. Both negative and weakly positive results have been obtained using human cells, sometimes within the same research group [6]. Given the equivocal nature of the results obtained, viewed in combination with the results of feeding studies in animals using irradiated meats that failed to detect increases in the generation of cancers and birth defects, and the low concentration of the 2-ACBs in irradiated meats, it is difficult to conceive that 2-ACBs present a significant risk to human health.

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